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Gonadotropin-Releasing Hormone (GnRH) Receptor Expression and Membrane Signaling in Early Embryonic GnRH Neurons: Role in Pulsatile Neurosecretion

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The characteristic pulsatile secretion of GnRH from hypothalamic neurons is dependent on an autocrine interaction between GnRH and its receptors expressed in GnRH-producing neurons. The ontogeny and function of this autoregulatory process were investigated in studies on the properties of GnRH neurons derived from the olfactory placode of the fetal rat. An analysis of immunocytochemically identified, laser-captured fetal rat hypothalamic GnRH neurons, and olfactory placode-derived GnRH neurons identified by differential interference contrast microscopy, demonstrated coexpression of mRNAs encoding GnRH and its type I receptor. Both placode-derived and immortalized GnRH neurons (GT1-7 cells) exhibited spontaneous electrical activity that was stimulated by GnRH agonist treatment. This evoked response, as well as basal neuronal firing, was abolished by treatment with a GnRH antagonist. GnRH stimulation elicited biphasic intracellular calcium ($[Ca^{2+}]_i$) responses, and both basal and GnRH-stimulated $[Ca^{2+}]_i$ levels were reduced by antagonist treatment. Perifused cultures released GnRH in a pulsatile manner that was highly dependent on extracellular Ca^{2+}. The amplitude of GnRH pulses was increased by GnRH agonist stimulation and was diminished during GnRH antagonist treatment. These findings demonstrate that expression of GnRH receptor, GnRH-dependent activation of Ca^{2+} signaling, and autocrine regulation of GnRH release are characteristics of early fetal GnRH neurons and could provide a mechanism for gene expression and regulated GnRH secretion during embryonic migration. (*Molecular Endocrinology* 18: 1808–1817, 2004)

THE GNRH NEURONS that determine reproductive processes in mammals originate in the embryonic olfactory placode and migrate into the forebrain during fetal development (1, 2) to form the hypothalamic pulse generator, which controls the pulsatile release of GnRH at the median eminence (3). Episodic GnRH release is essential for the maintenance of optimal gonadotropin secretion (4–7) and, consequently, for normal reproductive function in mammalian species (8, 9). Pulsatile neuropeptide secretion is an intrinsic property of the GnRH neuron and occurs in GnRH neurons cultured from the embryonic olfactory placode of rhesus monkeys (10), nasal placode cultures from embryonic rats (11), sheep olfactory explants (12), cultured rat fetal hypothalamic cells (13, 14), and immortalized GnRH neurons (15–17).

Episodic GnRH release from perifused hypothalamic cultures and immortalized GnRH neurons is highly dependent on extracellular Ca^{2+} (14–18). Furthermore, GnRH secretory profiles are influenced by the regulatory actions of GnRH agonist and antagonists on endogenous GnRH receptors expressed in GnRH neurons, with modulation of GnRH pulse frequency and amplitude (14). In this study on the ontogeny of the GnRH pulse generator, differential interference contrast (DIC) microscopy was used to characterize the morphological properties of cultured fetal GnRH neurons derived from the olfactory placode. The identity of DIC-identified GnRH neurons was confirmed by the use of single-cell RT-PCR to detect GnRH mRNA expression. To determine whether native GnRH-producing neurons coexpress transcripts for GnRH and the GnRH receptor (GnRH-R), laser capture microdissection was employed. Calcium signaling in DIC-identified GnRH neurons was monitored in fura-2-loaded neurons before and during treatment with GnRH agonist and antagonist analogs. The roles of Ca^{2+} entry and Ca^{2+} mobilization from intracellular stores in pulsatile GnRH release were investigated by activation and inhibition of voltage-gated Ca^{2+} channels and Ca^{2+}-mobilizing GnRH-Rs in cultured embryonic d 13 (E13) olfactory placode cells. These studies have revealed that pulsatile neurosecretory activity and autocrine regulation of neuronal firing and secretion by endogenous and exogenous GnRH are already operational in placode-

Abbreviations: AP, Action potential; $[Ca^{2+}]_i$, intracellular calcium; DIC, differential interference contrast; E13, embryonic d 13; GnRH-R, GnRH receptor.

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derived GnRH neurons. These findings indicate that GnRH neurons are functionally active and exhibit episodic neurosecretion before their migration into the brain to form the GnRH pulse generator network within the hypothalamus.

RESULTS

Expression of GnRH and GnRH-R and Morphological Identification of GnRH Neurons

Analysis of total RNA prepared from olfactory placode tissue, olfactory placode cells immediately after dispersion, and cultured olfactory placode cells, using gene-specific primers based on the sequences of the rat GnRH, gave the expected fragment size of 276 bp (Fig. 1A, lanes 2, 3, and 4, respectively). No amplification of GnRH mRNA was observed in tissue from the midbrain of E13 fetuses (Fig. 1A lane 1). DNA sequencing of the purified bands confirmed the authenticity of these amplified fragments, the nucleotide sequences of which matched those of the mouse GnRH gene (data not shown). These results demonstrated that the GnRH gene is expressed in olfactory placode tissue, placode cells immediately after dispersion, and cultured olfactory placode cells.

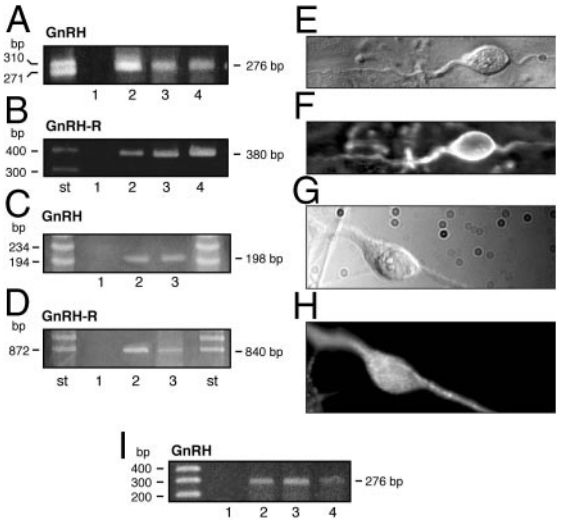

Fig. 1. Expression of GnRH and GnRH-R Transcripts and Morphological Properties of Cultured GnRH Neurons

A, Absence of GnRH expression in E13 midbrain tissue (lane 1). Detection of GnRH in fetal olfactory placode tissue (lane 2), olfactory placode cells immediately after enzymatic dispersion (lane 3), and cultured olfactory placode cells (lane 4). B, Absence of GnRH-R mRNA transcript in E13 midbrain tissue (lane 1). Expression of GnRH-R in the same samples shown in panel A. C, Absence of GnRH mRNA amplification in cultured GnRH-negative cells (lane 1). Expression of GnRH in laser-captured GT1-7 cells (lane 2) and native GnRH neurons (lane 3). D, Absence of GnRH-R expression in GnRH-negative cells. Detection of GnRH-R in laser captured GT1-7 cell (lane 2) and native GnRH neuron (lane 3). E and G, Morphology of two categories of cultured E13-derived GnRH neurons under DIC illumination. F and H, GnRH immunofluorescent staining of DIC-identified GnRH neurons. I, Expression of GnRH mRNA in DIC-identified E13-derived GnRH neurons. Lane 1, GnRH-negative olfactory cell. Lanes 2–4, individual, GnRH-positive olfactory neurons.

Furthermore, GnRH-R transcripts were coexpressed with GnRH in olfactory placode tissue and dispersed placode cells, as well as in cultured placode cells (Fig. 1B, lanes 2, 3, and 4, respectively). The amplified products were the expected size for GnRH-R (380 bp), and the nucleotide sequences matched the published sequences for mouse GnRH-R type I. No amplification of a specific GnRH-R product was observed in total RNA extracted from the midbrain of E13 fetuses (Fig. 1B, lane 1).

Laser microdissection of GnRH-immunostained cells was also used to analyze the expression of GnRH and GnRH-R transcripts in native hypothalamic GnRH neurons. GnRH immunoreactive cells were localized and positioned by direct microscopic visualization and then targeted by a laser pulse to induce cell transfer to thermoplastic film by local adhesion. RT-PCR of laser-captured cells using gene-specific primers revealed the expression of GnRH transcripts. Amplified products showed the expected size for GnRH (198 bp) in total RNA extracted from 25 pooled immortalized GnRH neurons and 25 GnRH-immunoreactive cultured hypothalamic cells (Fig. 1C, lanes 2 and 3, respectively). Amplification of total RNA extracted from 25 pooled GnRH-negative cells did not show a GnRH-specific product (Fig. 1C, lane 1). In the same RNA samples, GnRH-R transcripts were amplified with primers specific for mouse GnRH-R type I (Fig. 1D, lane 2, GT1-7 neurons; lane 3, GnRH-immunoreactive hypothalamic cells). No specific GnRH-R product was detected in cultured GnRH-negative hypothalamic cells (Fig. 1D, lane 1).

Under DIC illumination, two categories of bipolar GnRH neurons were identified based on nuclear size and localization. One type of GnRH neuron has a large and centrally placed nucleus, which contains an apparent nucleolus with subpolar localization. The nucleus is surrounded by a thin rim of cytoplasm, which extends throughout the processes (Fig. 1E). The other type of GnRH neuron was characterized by the presence of a relatively large nucleus that occupied 50% of the cross-sectional area of the cell body. In these cells, the cytoplasm contained numerous granules. Both categories of cells showed well-developed thick and quite long processes (Fig. 1G). The identity of such DIC-characterized cells was confirmed by immunofluorescent labeling for GnRH (Fig. 1, F and H). Expression of GnRH transcripts was found by single-cell RT-PCR in more than 95% of DIC-identified GnRH neurons, confirming the accuracy of the morphological criteria used to identify cultured olfactory placode GnRH neurons. The fragments of amplified GnRH mRNA products from three individual GnRH neurons are shown in Fig. 1I, lanes 2, 3, and 4). No GnRH

mRNA product amplification was detected in single cells that did not morphologically resemble GnRH neurons (Fig. 1I, lane 1).

Immunostaining of olfactory placode cells fixed immediately after dispersion with a specific polyclonal GnRH antibody, and a GnRH-R antibody (raised against sequences of the third intracellular loop and sixth transmembrane domain of the mouse GnRH-R; residues 234–298) revealed that 4.7 ± 1.6% (of 4978 cells in three experiments) were GnRH positive and 3.4 ± 1.9% (of 5794 cells in three experiment) showed GnRH-R staining. The GnRH-positive cells counted in dispersed olfactory placodes probably include numerous progenitor cells and differentiating GnRH neurons and do not represent the number of differentiated GnRH neurons. Differentiated olfactory placode-derived GnRH neurons with typical bipolar morphology were seen after 1 wk of culture. The GnRH-immunoreactive product was uniformly distributed throughout the cytoplasm as well as in primary processes and was absent from the nucleus. The immunoreactive product characteristic of GnRH-R immunostaining was localized at the plasma membrane of bipolar neurons and occasionally also on unidentified round cells. Double immunostaining revealed that the majority of the typical bipolar GnRH neurons also exhibited specific staining for the GnRH-R (19).

Modulation of Spontaneous Firing of Action Potentials (APs) by GnRH Agonist and Antagonist Analogs

Firing of APs in placode-derived GnRH neurons and GT1-7 neurons was monitored by the cell-attached technique in the current-clamp mode. Data were obtained from single isolated GnRH neurons to eliminate the influences of electrical and synaptic coupling between cells. Under these recording conditions, both native and immortalized GnRH neurons exhibited spontaneous AP firing. The firing frequency ranged from high (0.8–1.2 Hz) in 25% of cells, to moderate (0.3–0.5 Hz) in 48% of cells, and low (<0.1 Hz) in 10% of cells; about 17% of 56 native GnRH neurons were silent. Very similar firing properties were observed in GT1-7 neurons (Fig. 2, A and A′, and G and G′). Most of the cells (77%) showed irregular spiking activity. However, 6% showed bursting activity separated by quiescent periods of 5–10 sec, similar to that previously observed in native GnRH neurons (20, 21). GnRH treatment (100 nM) caused a significant increase in the frequency of AP firing, from basal (0.6 ± 0.05 Hz to 1.1 ± 0.9 Hz, $P < 0.01$; n = 12) in native GnRH neurons, and from basal (0.8 ± 0.08 Hz to 1.9 ± 0.2, Hz $P < 0.01$; n = 8) in GT1-7 neurons (Fig. 2, B and E.

In both native and GT1-7 neurons, GnRH agonist treatment changed the pattern of AP firing to a more complex form, often with bifurcated peaks (Fig. 2, B′ and E′). The GnRH-induced increase in AP frequency was almost abolished during concomitant treatment with CDB-3883H, a potent GnRH antagonist, and only occasional APs were recorded (Fig. 2, C and C′ and F and F′). The characteristic basal firing of APs recovered during the washing period (Fig. 2, D and H). In addition to suppressing GnRH agonist-induced APs, the CDB antagonist also abolished spontaneous firing of APs in cultured GnRH neurons. The antagonist-induced inhibition of spontaneous electrical activity (Fig. 3A) was time dependent, and AP firing progressively decreased and was abolished over 7.5 min (Fig. 3, B–F). Spontaneous AP firing resumed during washout of the antagonist and returned to the control level after 3 min (Fig. 3, G and H).

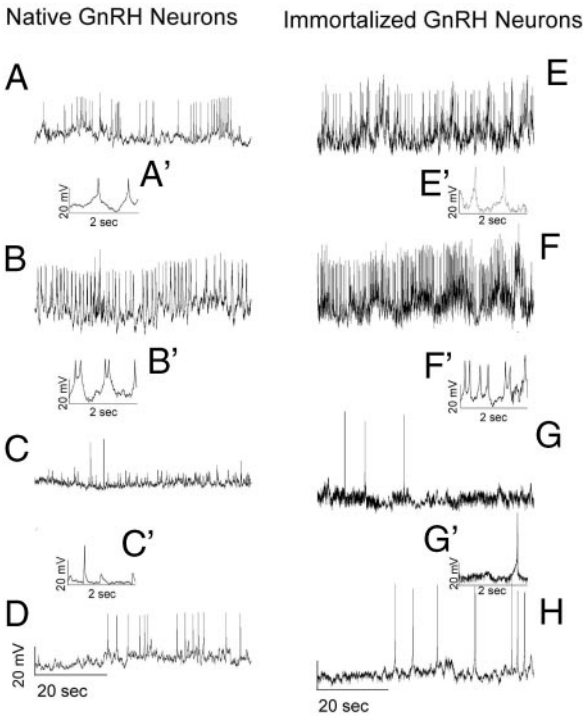

Fig. 2. Modulation of Spontaneous Electrical Activity of Cultured DIC-Identified E13 Olfactory Placode-Derived and Immortalized GnRH Neurons

A, Basal electrical activity (1-min recording) in cell-attached mode of cultured olfactory placode-derived GnRH neurons. A′, Enlarged basal APs. B, Stimulatory action of 100 nM GnRH on neuronal firing of the same GnRH neuron (1-min recording). B′, enlarged GnRH-induced APs. C, Inhibition of GnRH-stimulated firing by concomitant application of GnRH antagonist analog to the same GnRH neuron (1-min recording). C′, Enlarged APs during GnRH and GnRH antagonist application. D, Recovery of spontaneous electrical activity during antagonist washout. E and E′, Basal electrical activity of GT1-7 neurons. G and G′, Stimulatory action of GnRH on AP firing in GT1-7 neurons. F and F′, Inhibition of AP firing by GnRH antagonist analog. H, Recovery of spontaneous electrical activity during antagonist washout.

Calcium Signaling

In DIC-identified GnRH neurons loaded with the calcium-sensitive dye, fura-2, 25% of 32 examined GnRH neurons showed basal cytosolic calcium

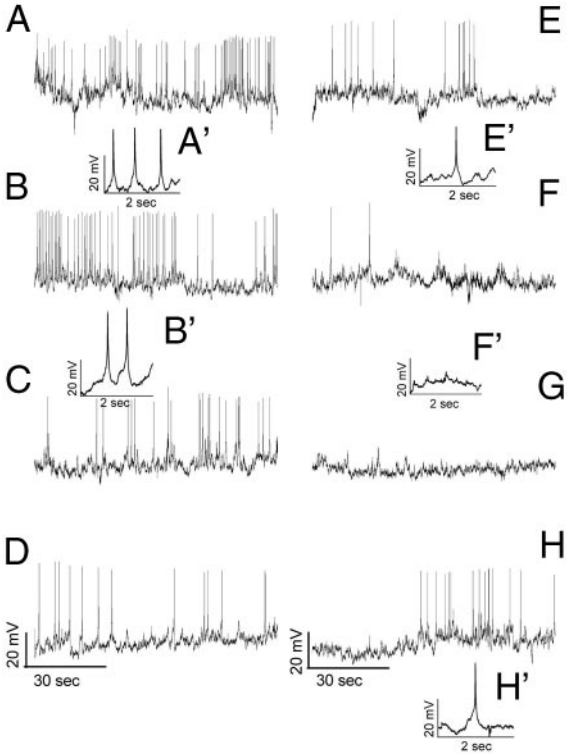

Fig. 3. Inhibition of Spontaneous Electrical Activity in Cultured DIC-Identified E13 Olfactory Placode-Derived GnRH Neurons by GnRH Antagonist Treatment

A, Firing of spontaneous APs in cultured GnRH neuron. A′, Enlarged basal APs. B and B′, Time-dependent inhibition of spontaneous AP firing of the same GnRH neuron during GnRH antagonist treatment (1.5 min). C, During 3 min of GnRH antagonist application; D, during 4.5 min; E and E′, during 6 min; F and F′, during 7.5 min. G, Recovery of the spontaneous electrical activity of the same GnRH neuron during washout of the GnRH antagonist (1.5 min); H and H′, after 3 min.

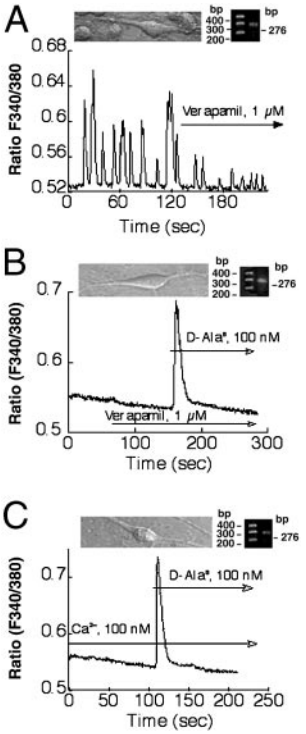

Fig. 4. Calcium Influx Dependence of Ca^{2+} Oscillations in DIC-Identified GnRH Neurons

A, Inhibition of spontaneous $[Ca^{2+}]_i$ oscillations by the Ca^{2+} channel antagonist, verapamil. B and C, Abolition of the GnRH-induced plateau phase in $[Ca^{2+}]_i$ response by verapamil treatment and extracellular calcium depletion, respectively.

($[Ca^{2+}]_i$) oscillations that were dependent on extracellular Ca^{2+} and were inhibited by the L-type channel blocker, verapamil (Fig. 4A). Treatment with verapamil also abolished the plateau phase elicited by GnRH agonist stimulation but did not change the initial spike phase (Fig. 4B). When the cells were bathed in Ca^{2+}-free medium, the $[Ca^{2+}]_i$ spike response to GnRH agonist stimulation was likewise unchanged, and the plateau phase was abolished (Fig. 4C). The identity of DIC-identified GnRH responding neurons was subsequently confirmed by single-cell RT-PCR, and the expression of GnRH transcripts is shown as an *inset* in the corresponding figures.

Calcium recordings from DIC-identified single GnRH neurons showed that activation of GnRH-Rs with 100 nM [D-Ala^6]Ag, a potent GnRH agonist, caused biphasic increases in $[Ca^{2+}]_i$ in all GnRH neurons studied. This response was characterized by a rapid and prominent Ca^{2+} spike followed by a plateau phase that gradually declined to near-basal levels (Fig. 5A). Immortalized GnRH neurons also responded to GnRH agonist stimulation with biphasic $[Ca^{2+}]_i$ responses that were similar to those of native GnRH neurons. The morphological characteristics of normal and immortalized GnRH neurons were very similar, and the two cell types were almost indistinguishable (Fig. 5B). The stimulatory actions of GnRH agonist analogs on $[Ca^{2+}]_i$ responses were abolished in the presence of the GnRH-R antagonist (Fig. 5C). Application of the antagonist also reduced the basal $[Ca^{2+}]_i$, which partially recovered during subsequent treatment with the agonist analog, [D-Ala^6]Ag (Fig. 5D).

Effects of GnRH Agonist and Antagonist Analogs on Pulsatile GnRH Release

Perifused olfactory placode tissue and olfactory placode cells cultured on cytodex beads consistently exhibited pulsatile GnRH release. Representative examples of pulsatile GnRH release in cultured olfactory placode tissues and cultured olfactory neurons are shown in Fig. 6, A and B, with several episodic increases in GnRH release during the 7-h sampling period. Each episodic increase was statistically defined as a GnRH peak, indicating that E13 cultured GnRH neurons released GnRH in a pulsatile manner. The estimated interpulse interval was 29.9 ± 1.6 min, and

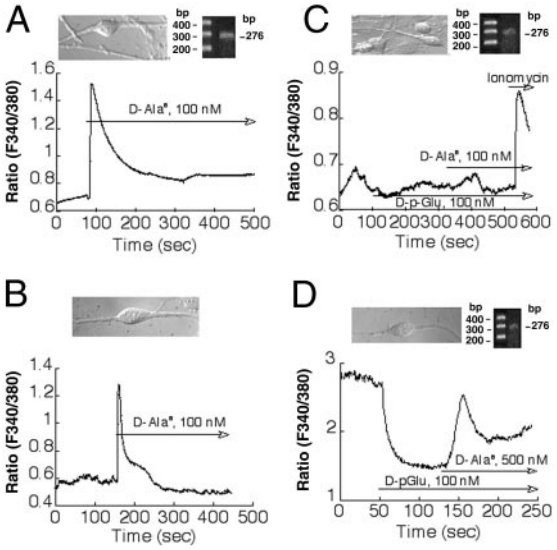

Fig. 5. Calcium Signaling in DIC-Identified GnRH Neurons

A and B, Typical biphasic $[Ca^{2+}]_i$ increases in response to GnRH agonist application to olfactory placode-derived and immortalized GnRH neurons. C, Absence of $[Ca^{2+}]_i$ response to GnRH agonist stimulation during concomitant GnRH antagonist treatment. D, Reduction of both basal $[Ca^{2+}]_i$ and GnRH-stimulated Ca^{2+} response in olfactory placode-derived GnRH neurons during GnRH antagonist treatment.

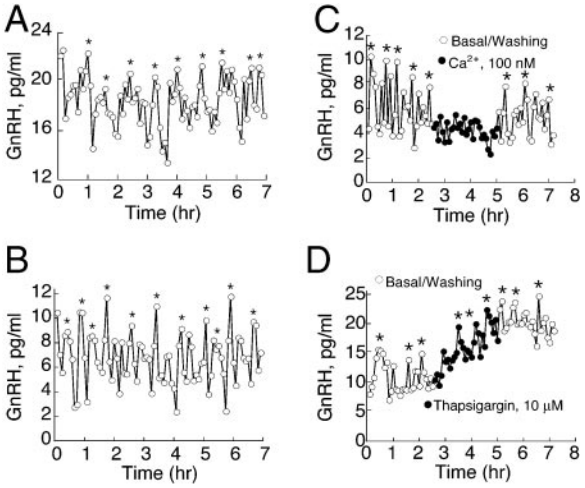

Fig. 6. Episodic GnRH Release in Cultured Olfactory Placode Cells

A and B, Representative examples of pulsatile GnRH secretion in olfactory placode tissue and cultured cells, respectively. GnRH pulses determined by cluster analysis are indicated by *asterisks*. C, Depletion of extracellular calcium reversibly inhibits pulsatile GnRH release. D, Release of stored intracellular Ca^{2+} by thapsigargin monotonically increases GnRH secretion.

the mean amplitude of the GnRH pulses was 11.5 ± 0.6 pg/ml (n = 15 independent experiments).

To determine the dependence of GnRH pulses on extracellular Ca^{2+} entry, olfactory placode cells were perifused in calcium-free medium. Under these conditions, the basal episodic release of GnRH was reversibly abolished (Fig. 6C). A similar effect on GnRH release was observed when cells were perifused with 1 μM verapamil (data not shown), indicating that Ca^{2+} entry through L-type voltage-sensitive calcium channels is required for basal pulsatile GnRH release. Transient depolarization of perifused olfactory cultures with 35 mM KCl to promote extracellular Ca^{2+} entry (data not shown), and mobilization of Ca^{2+} from intracellular stores by treatment with thapsigargin (Fig. 6D), also caused significant increases in GnRH release.

Activation of olfactory placode GnRH-Rs by exposure to 10 nM and 100 nM [D-Ala^6]Ag caused significant increases in both interpulse interval and pulse amplitude. The interpulse interval was extended from 29.9 ± 1.6 min (n = 15) in controls to 38.2 ± 1.5 min (n = 3) in10 nM [D-Ala^6]Ag-treated cells ($P < 0.05$; Fig. 7A). The average peak height was also increased, from 11.5 ± 0.6 pg/ml to 15.2 ± 0.9 pg/ml (n = 3) during the treatment with 10 nM [D-Ala^6]Ag ($P < 0.05$; Fig. 7A). Further increases in interpulse interval from 29.9 ± 1.6 min (n = 15) to 45.6 ± 3.5 min (n = 7) and peak amplitude from 11.5 ± 0.6 pg/ml to 17.2 ± 1.5 pg/ml (n = 7) were observed during treatment with 100 nM [D-Ala^6]Ag ($P < 0.01$, Fig. 7B). In contrast, perifusion of olfactory placode cells with the GnRH-R antagonist, CDB-3883H, which does not cross-react in the GnRH RIA (Fig. 7H), caused dose-dependent reversible cessation of pulsatile GnRH release. An initial reduction of pulse frequency by 12.5 ± 2.3% [1.6 ± 0.2 peaks/h control to 1.4 ± 0.2 peaks/h (n = 3)] was observed during treatment with 1 nM CDB (data not shown). The pulse frequency progressively decreased by 62 ± 8.4% [1.6 ± 0.2 peaks/h control to 0.6 ± 0.1 peaks/h during treatment with 10 nM CDB (Fig. 7, C and F, $P < 0.05$; n = 3)], and by 85 ± 10.6% [1.6 ± 0.2 peaks/h control to 0.24 ± 0.04 peaks/h 100 during treatment with 100 nM CDB (Fig. 7, D and F, $P < 0.05$; n = 3)], and 92 ± 12.3% [1.6 ± 0.2 peaks/h control to 0.12 ± 0.02 peaks/h during treatment with 1 μM CDB (Fig. 7, E and F, $P < 0.05$; n =3)]. Treatment of perifused olfactory placode cells with a nonpeptide GnRH-R antagonist also reversibly inhibited pulsatile GnRH release (Fig. 7G).

DISCUSSION

Episodic hormone secretion is a characteristic feature of the hypothalamo-pituitary-gonadal system, in which the profile of gonadotropin release from pituitary gonadotrophs reflects the pulsatile secretory activity of GnRH-producing neurons in the hypothalamus (22, 23). Pulsatile release of GnRH is also evident *in vitro* during perifusion of cultured fetal hypothalamic cells, which produce bioactive GnRH for up to 2 months *in vitro*. Such cultures, as well as hypothalamic tissue from adult rats, were found to express GnRH-Rs as evidenced by the presence of high-affinity GnRH binding sites and GnRH-R transcripts (14). The ability of

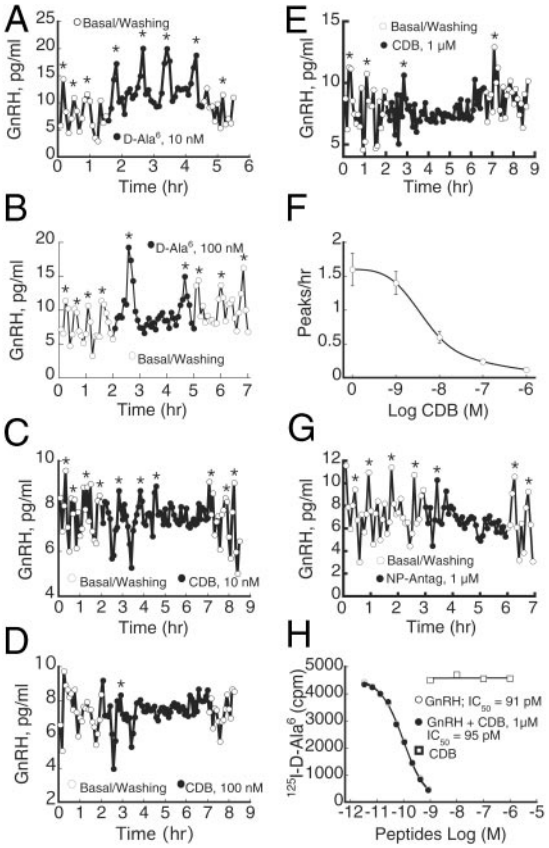

Fig. 7. Modulation of Pulse Amplitude and Frequency in Cultured Olfactory Placode Neurons by GnRH Agonist and Antagonist Analogs

A and B, Increase of peak amplitude and interpulse interval during GnRH agonist treatment (10 nM and 100 nM [D-Ala^6]Ag). C–E, Progressive inhibition of pulsatile GnRH secretion by antagonist treatment (10 nM to 1 μM CDB). F, Dose-dependent inhibition of pulsatile GnRH release by CDB. G, Inhibition of pulsatile GnRH secretion by nonpeptide GnRH-R antagonist (NP-Antag). H, Lack of cross-reactivity of CDB peptide GnRH antagonist in the GnRH RIA.

immortalized GnRH neurons (GT1-7 cells) to exhibit episodic GnRH release in the absence of any other cell types suggests that intrinsic factors, such as autocrine regulation of neurosecretion, are important determinants of pulsatile GnRH release. The findings that GT1-7 cells express GnRH-R, and that agonist activation modulates pulsatile GnRH release by changing pulse frequency and amplitude (14, 24), are consistent with this proposal.

In the present studies, E13 olfactory placode GnRH neurons were identified by morphological criteria under DIC illumination with greater than 95% accuracy. The bipolarity, position of nucleus, appearance of nucleolus, and relationship between nucleus and cytoplasm are reliable features in the identification of GnRH neurons. We also observed that the morphological characteristics of differentiated immortalized GnRH neurons are similar to those of normal GnRH neurons. When cultured at low density, such GT1-7 neurons form a neuronal network that resembles the GnRH neuronal network observed in mixed populations of cultured hypothalamic cells.

DIC-identified E13 GnRH neurons express GnRH mRNA, as determined by single-cell RT-PCR and laser capture microdissection, and coexpress transcripts for the GnRH-R. Expression of both GnRH and GnRH-R transcripts in early embryonic olfactory placode-derived GnRH neurons provides an autoregulatory system that may operate *ab initio* to maintain cellular activity and neurosecretion during neuronal migration into the forebrain en route to the hypothalamus.

Spontaneous firing of APs in the cell-attached current-clamp mode was evident in both native and immortalized GnRH neurons and was similar to that observed in olfactory placode-derived GnRH neurons (25), as well as GnRH neurons expressing green fluorescent protein (20, 21, 26), and immortalized GnRH neurons (27–30). In our study, GnRH treatment of both native and GT1-7 neurons consistently increased the frequency of AP firing. Such enhanced AP firing was GnRH-R dependent, and was diminished by GnRH antagonist treatment. Furthermore, the basal firing of APs in GnRH neurons was also GnRH-R dependent and was abolished during GnRH antagonist treatment. Basal electrical activity returned during washout of the antagonist, indicating that the GnRH autoregulatory system participates in the control of spontaneous APs firing in both native and GT1-7 neurons. Cultured native GnRH neurons and immortalized GnRH neurons have closely similar electrical properties, and GT1-7 neurons can serve as a surrogate for native neurons in electrophysiological studies. Also, green fluorescent protein-tagged GnRH neurons of adult castrated male mice express GnRH-Rs, and GnRH treatment exerts both stimulatory and inhibitory effects on AP firing (31).

DIC-identified GnRH neurons loaded with the Ca^{2+} indicator, fura-2, responded to GnRH treatment with rapid and prominent increases in $[Ca^{2+}]_i$, followed by a sharp decrease and a prolonged plateau phase. The spike phase was GnRH-R dependent and extracellular Ca^{2+} independent and was unchanged in Ca^{2+}-free medium and during blockade of extracellular Ca^{2+} entry by verapamil, consistent with Ca^{2+} mobilization from intracellular stores. In contrast, the plateau phase was dependent on GnRH-R activation and extracellular Ca^{2+}. Similar $[Ca^{2+}]_i$ profiles were elicited by the Ca^{2+}-mobilizing GnRH-Rs expressed in pituitary gonadotrophs (32), immortalized pituitary gonadotrophs (αT3-1) (33), and GT1-7 neurons (17). Basal $[Ca^{2+}]_i$ oscillations were Ca^{2+} dependent and were abolished during treatment with verapamil, and during extracellular Ca^{2+} depletion. In native GnRH neurons, basal $[Ca^{2+}]_i$ was also GnRH-R dependent and was diminished during blockade of autocrine GnRH-R activity by the GnRH antagonist analog. These findings demonstrate that cultured E13-derived GnRH neurons express functional GnRH-Rs and exhibit $[Ca^{2+}]_i$ responses resembling those of homogenous populations of immortalized GnRH neurons. The notable similarity in $[Ca^{2+}]_i$

responses of identified GnRH neurons in cultured olfactory placode cells and GT1-7 neurons indicates that differentiated GT1-7 neurons provide a useful model in which to investigate Ca^{2+} and other signaling pathways that characterize the native GnRH neuron.

In our experiments, electrical recordings and single-cell Ca^{2+} measurements were obtained from morphologically differentiated cells, cultured in defined medium without fetal calf serum for at least 5 d. In contrast, $[Ca^{2+}]_i$ spiking is more frequent in proliferating GT1-1 cells (27, 34, 35) and may reflect differences in ion channel expression and conductivity, as well as the formation of gap junctions between cell bodies (30, 36).

As previously observed in hypothalamic GnRH neurons and GT1-7 cells, GnRH antagonist analogs abolished pulsatile GnRH secretion from perifused olfactory placode-derived cells. In contrast, GnRH agonist treatment reduced the frequency and increased the amplitude of pulsatile GnRH release. These findings are consistent with the proposal that autocrine activation of GnRH-Rs in cultured olfactory placode-derived GnRH neurons is required for pulsatile GnRH release *in vitro*. The effects of GnRH agonist and antagonist analogs on neuropeptide release are also consistent with the operation of an ultrashort loop feedback mechanism that exerts both positive and negative actions, which contribute to the integrated control of neuropeptide secretion from GnRH neurons (14).

The expression of GnRH and GnRH-R mRNA in E13 olfactory placode tissue and olfactory placode cells obtained immediately after dispersion, and the pulsatile GnRH release from perifused E13 olfactory placodes, demonstrates that the autocrine GnRH regulatory system previously observed in hypothalamic neurons is also operative in the earliest identifiable GnRH neurons. It is possible that activation of GnRH-R signaling and gene induction previously observed in GT1-7 cells (37) and LβT2 gonadotrophs (38) and during migration of human olfactory placode-derived cells (FNC-B4) (39), promotes the expression of genes that are relevant to the tracking and secretory properties of immature GnRH neurons. At this stage, the developing neurons presumably lack the neuronal and hormonal inputs that regulate pulsatile GnRH release at their final destination in the brain. Within the hypothalamus, the autoregulatory control of GnRH neuronal activity becomes integrated with other neuronal and hormonal inputs to provide a more complex control system with a high degree of redundancy to drive and maintain pulsatile GnRH release and reproductive function.

MATERIALS AND METHODS

Animals and Olfactory Placode Dispersion

Olfactory placodes were removed from fetuses of 13-d pregnant Sprague Dawley rats (Charles River, Wilmington, MA) under a dissection microscope and placed in ice-cold dissociation buffer (0.01 M PBS containing 1 mg/ml glucose, pH 7.4). Before enzymatic digestion, the tissue was washed and incubated in a sterile flask with dissociation buffer supplemented with 0.1% collagenase type II (activity 305 U/mg; Worthington Biochemical Corp., Freehold, NJ) and 0.1% DNAse (Sigma Chemical Co., St. Louis, MO). After 15 min of gentle shaking, the tissue was disrupted by repeated aspiration into a smooth-tipped Pasteur pipette. Incubation was continued for another 15 min, and tissue was again dispersed. The cells were sedimented by centrifugation for 10 min at 200 $\times$ *g*, washed once in dissociation buffer, and once in regular culture medium consisting of DMEM/Ham's F-12 medium (1:1 vol/vol), 3.2 g/liter glucose, 2.5 g/liter sodium bicarbonate, 100 μg/ml gentamicin, B-27 supplement, and 10% heat-inactivated fetal bovine serum (Life Technologies, Gaithersburg, MD). Cellular viability, as estimated by the trypan blue exclusion test in a Neubauer chamber, was higher than 95%.

Cell Culture

Dispersed cells were plated at a density of 200,000 cells/ml on collagen-coated 25-mm coverslips (for patch-clamp, calcium measurements, and immunocytochemistry) and allowed to attach for 45 min in 25-mm culture dishes. After initial attachment, an additional 1 ml of culture medium was added and the cells were cultured for 48 h, followed by serum-free medium supplemented with B-27. Cultures were maintained for 7–14 d before patch-clamp, calcium measurements, or immunocytochemistry, with change of medium every 48 h.

For perifusion experiments, 10^7 olfactory placode cells were incubated for 30 min in 0.3 ml of preswollen cytodex-2 beads (Pharmacia Biotech, Piscataway, NJ) to allow attachment of cells, followed by addition of 2 ml culture medium. Cultures were maintained for 7–10 d before perifusion, and the medium was changed every 2 d. Such cultures initially contained diverse cell types, and proliferation of nonneuronal cells was minimized by the use of serum-free defined medium. Single olfactory placodes yielded approximately 1×10^6 cells. The cells were fixed immediately after dispersion and stained for GnRH and GnRH-R. The number of GnRH-positive cells was counted in three separate experiments and of a total 4978 cells, 4–4.7% were GnRH positive. For perifusion studies 1×10^7 olfactory placode cells were plated on cytodex beads, giving an estimated 400,000–470,000 of GnRH-positive cells per perifusion chamber. The placode cells are cultured on cytodex beads for at least 1 wk before perifusion studies, and it has not yet been possible to obtain an accurate count of the attached differentiated GnRH neurons at the end of this time.

Immortalized GnRH neurons (GT1-7 cells) (40), kindly provided by Dr. Richard I. Weiner (University of California, San Francisco, CA), were grown in culture medium containing (DMEM/F-12, 1:1, with L-glutamate, pyridoxine hydrochloride, 2.5 g/liter sodium bicarbonate, 10% heat-inactivated fetal bovine serum, and 100 μg/ml gentamicin; Life Technologies). For electrophysiological recordings and single-cell calcium measurement, confluent GT1-7 neurons were dispersed by trypsinization (0.05% trypsin) for 10 min, resuspended in culture medium, and plated (15,000 cells/ml) in 35-mm poly-L-lysine-coated (0.01%) coverslips. After incubation for 48 h, the culture medium was replaced with medium containing B-27 serum-free supplement (Life Technologies) to induce morphological differentiation of the cells. All experiments were performed 5–7 d after removal of serum.

Laser Capture of GnRH-Immunostained Cells

For laser capture microdissection experiments, cells were plated at a low density on grid glass coverslips, fixed in 3%

paraformaldehyde, and then dehydrated and kept dry at −70 C until further use. After immunostaining and localization of GnRH-immunopositive cells, single-cell microdissection was performed using a PixCell laser capture microscope (Arcturus Engineering, Santa Clara, CA) as previously described (41, 42). Briefly, a clear transfer film mounted on an optically transparent cap was placed on top of the coverslips. Immunoreactive cells were captured by targeting and activating an infrared laser beam, which locally melted the thermoplastic film and fused into the underlying cell of choice. The captured cells were immersed in RNA isolation reagent (Trizol reagent from Life Technologies). Captured nonimmunoreactive cells were used as negative controls.

Single-Cell RNA Harvesting and RT-PCR

Neurons were collected with a glass pipette containing 10 mM deoxynucleotide triphosphate mix, Oligo(dT)$_{12-18}$ (0.5 μg/μl), and RNase guard (20 U). Immediately after harvesting, the cell content was expelled from the pipette by brief centrifugation and incubated at 65 C for 5 min. RNA was reverse transcribed to cDNA by using SuperScript II RNase H-reverse transcriptase (Life Technologies) following the manufacturer's instructions. Finally, the reverse transcriptase reaction was inactivated by incubation for 10 min at −70 C.

Immunostaining and Morphology of GnRH Neurons

To determine the morphological features of GnRH neurons, cultured cells from E13 olfactory placodes were immunocytochemically stained for GnRH content using fluorescent secondary antibody. Morphological characteristics of the identified GnRH neurons were analyzed on an upright microscope (Olympus Corp., Lake Success, NY) fitted with epifluorescence illumination and DIC optics.

GnRH and GnRH-R Single-Cell PCR Amplification

PCRs were performed in 20 μl final volume following the LightCycler protocol using FastStart DNA Master SYBR green I (master mixes supplied in kits from Roche Diagnostics, Indianapolis, IN) by adding 10 μl reverse transcription (RT) product, 3 mM Mg^{2+}, 0.5 μM GnRH primer [(rat, NM 012767; 1–21) 5′-ATGGAAACGATCCCCAAACTG-3′ and (274–253) 5′-CATCTTCTTCTGCCCAGCTTCC-3′] for 35 cycles; and 3 mM Mg^{2+}, 0.3 μM GnRH-R primers [(rat, L25053; 614–636) 5′-CAGTCTTCTCGCAATGTGTGACC-3′ and (<993–972) GCACGGGTTTAGGAAAGCAAAG-3′] for 30 cycles. Temperature profiles for GnRH: 94 C/1 sec, 65 C/7 sec, and 72 C/12 sec for 35 cycles. Temperature profiles for GnRH-R: 94 C/1 sec, 60 C/7 sec, and 72 C/16 sec for 30 cycles. The GnRH and GnRH-R cDNA fragments were extracted from gels using the QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA), and the identities of PCR products were confirmed by sequencing.

Cell-Attached Patch-Clamp Recording

Current-clamp recordings were performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) and were low-pass filtered at 2 kHz. The spontaneous membrane activities of cells were recorded in the cell-attached mode. This method of measuring spontaneous membrane activities minimizes disturbance of the intracellular contents and provides optimal conditions for recording APs. In this mode, APs appear as brief upward and/or downward spikes. Electrodes used for patch recordings were pulled from borosilicate glass (1.5-mm outer diameter; World Precision Instruments, Sarasota, FL) with a Flaming Brown horizontal puller (P-87, Sutter Instruments, Novato, CA), and then heat-polished to a final tip resistance of 3.5–5.5 MΩ. Digidata 1320A analog-to-digital interface, Clampex 9 (Axon Instruments) software were used for data acquisition and analysis. For recording of membrane electrical activity, the bath and pipette solutions were the same composition as normal extracellular solution (in mM): 142 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 3 KCl, 10 glucose, and 10 HEPES, pH-adjusted to 7.4 with NaOH. The culture chambers were continuously perfused at a flow rate of 1 ml/min with the above solution at room temperature (22 C).

Single-Cell Calcium Measurements

Cells were incubated for 30 min at 37 C with 0.5 μM Fura-2 AM (Molecular Probes, Eugene, OR) in phenol red-free DMEM containing 20 mM $NaHCO_3$, pH 7.4. Coverslips were washed with phenol red-free Krebs-ringer buffer and mounted on the stage of an Olympus IX-70 microscope with attached MicroMax 5 MHz digital camera. Cells were examined under a ×40 oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. Changes in $[Ca^{2+}]_i$ were derived as ratios of the latest two excitation wavelengths (F340/F380) and converted to concentration units using the Grynkiewicz equation (43). Data were obtained at a sampling rate of two points per sec using Axon Imaging Workbench 2.2.

Cell Perifusion Procedure

The bead-attached cells were collected by gravity sedimentation and placed in multiple microchamber modules (Cellex Biosciences, Inc., Minneapolis, MN) with an internal volume of 0.5 ml. Cells were perifused at 37 C with DMEM/F12 (1:1) containing 0.1% BSA and 15 mM HEPES at a flow rate of 0.13 ml/min. Fractions were collected at 5-min intervals and stored at −20 C before RIA of GnRH content using [^{125}I]GnRH (Amersham Pharmacia Biotech, Arlington Heights, IL) as tracer. The intra- and interassay coefficients of variation at 80% binding in standard samples (15 pg/ml) were 12% and 14%, respectively. The sensitivity of the RIA, defined as twice the SD at the zero dose, was 0.2 pg/tube. GnRH agonist (des-Gly^{10}-[D-Ala^6]GnRH *N*-ethylamide; [D-Ala^6]Ag), and antagonist ([D-pGlu^1, D-Phe^2, D-Trp^{3,6}]GnRH; [D-pGlu]Antag) analogs were from Peninsula Laboratories (Belmont, CA). The potent GnRH antagonist, acyline [CDB 3883H (Acetyl-D-Nal-D-4-Cl-Phe-D-Pal-Ser-Aph(Ac)-D-Aph(Ac)-Leu-Lys(Ipr)-Pro-DAlaNH2)] was provided by Dr. Hyun Kim (National Institutes of Health, Bethesda, MD). The nonpeptide GnRH antagonist, 1H-quinoline, was a gift from Dr. James Schaeffer, Merck Research Laboratories (Rahway, NJ) (44). There was no detectable cross-reactivity of the GnRH agonist or antagonist analogs used in the GnRH RIA.

Data Analysis

GnRH pulses and their parameters were identified and determined by a computer-based algorithm cluster analysis (45). Individual point standard derivations were calculated using a power function variance model from the experimental duplicates. A 2 × 2 cluster configuration and a *t* statistic of 2 for upstroke and downstroke were used to maintain false positive and negative error rates below 10%. The statistical significance of the pulse parameters and hormone levels was tested by repeated measures ANOVA. Duncan's multiple range test with critical range level of 0.01 was used as a *post hoc* test.

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REFERENCES

1. Schwanzel-Fukuda M 1999 Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc Res Tech 44:2–10
2. Wray S 2002 Development of gonadotropin-releasing hormone-1 neurons. Front Neuroendocrinol 23:292–316
3. Knobil E 1990 The GnRH pulse generator. Am J Obstet Gynecol 163:1721–1727
4. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF 1971 Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science 173:1036–1038
5. Marshall JC, Kelch RP 1986 Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. N Engl J Med 315:1459–1468
6. Levine JE 1997 New concepts of the neuroendocrine regulation of gonadotropin surges in rats. Biol Reprod 56:293–302
7. Krsmanovic LZ, Stojilkovic SS, Catt KJ 1996 Pulsatile gonadotropin-releasing hormone release and its regulation. Trends Endocrinol Metab 7:56–59
8. Crowley Jr WF, Filicori M, Spratt DI, Santoro NF 1985 The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res 41:473–531
9. Knobil E 1988 The hypothalamic gonadotrophic hormone releasing hormone (GnRH) pulse generator in the rhesus monkey and its neuroendocrine control. Hum Reprod 3:29–31
10. Terasawa E, Keen KL, Mogi K, Claude P 1999 Pulsatile release of luteinizing hormone-releasing hormone (LHRH) in cultured LHRH neurons derived from the embryonic olfactory placode of the rhesus monkey. Endocrinology 140:1432–1441
11. Funabashi T, Daikoku S, Shinohara K, Kimura F 2000 Pulsatile gonadotropin-releasing hormone (GnRH) secretion is an inherent function of GnRH neurons, as revealed by the culture of medial olfactory placode obtained from embryonic rats. Neuroendocrinology 71:138–144
12. Duittoz AH, Batailler M 2000 Pulsatile GnRH secretion from primary cultures of sheep olfactory placode explants. J Reprod Fertil 120:391–396
13. Krsmanovic LZ, Stojilkovic SS, Balla T, al-Damluji S, Weiner RI, Catt KJ 1991 Receptors and neurosecretory actions of endothelin in hypothalamic neurons. Proc Natl Acad Sci USA 88:11124–11128
14. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Navarro CE, Chen HC, Stojilkovic SS, Catt KJ 1999 Autocrine regulation of gonadotropin-releasing hormone secretion in cultured hypothalamic neurons. Endocrinology 140:1423–1431
15. Martinez de la Escalera G, Choi AL, Weiner RI 1992 Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. Proc Natl Acad Sci USA 89:1852–1855
16. Wetsel WC, Valenca MM, Merchenthaler I, Liposits Z, Lopez FJ, Weiner RI, Mellon PL, Negor-Vilar A 1992 Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. Proc Natl Acad Sci USA 89:4149–4153
17. Krsmanovic LZ, Stojilkovic SS, Merelli F, Dufour SM, Virmani MA, Catt KJ 1992 Calcium signaling and episodic secretion of gonadotropin-releasing hormone in hypothalamic neurons. Proc Natl Acad Sci USA 89:8462–8466
18. Terasawa E, Schanhofer WK, Keen KL, Luchansky L 1999 Intracellular Ca(2+) oscillations in luteinizing hormone-releasing hormone neurons derived from the embryonic olfactory placode of the rhesus monkey. J Neurosci 19:5898–5909
19. Martinez-Fuentes AJ, Krsmanovic LZ, Saito S, Chen H-C, Catt KJ, Expression and function of GnRH receptors in cultured rat E12 GnRH neurons. In: Program of the 82nd Annual Meeting of The Endocrine Society, Toronto, Canada, 2000, p 123 (Abstract 523)
20. Suter KJ, Song WJ, Sampson TL, Wuarin JP, Saunders JT, Dudek FE, Moenter SM 2000 Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. Endocrinology 141:412–419
21. Kuehl-Kovarik MC, Pouliot WA, Halterman GL, Handa RJ, Dudek FE, Partin KM 2002 Episodic bursting activity and response to excitatory amino acids in acutely dissociated gonadotropin-releasing hormone neurons genetically targeted with green fluorescent protein. J Neurosci 22:2313–2322
22. Phelps CP, Kalra SP, Kalra PS 1992 In vivo pulsatile LHRH release into the anterior pituitary of the male rat: effects of castration. Brain Res 569:159–163
23. Terasawa E 1998 Cellular mechanism of pulsatile LHRH release. Gen Comp Endocrinol 112:283–295
24. Krsmanovic LZ, Mores N, Navarro CE, Arora KK, Catt KJ 2003 An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion. Proc Natl Acad Sci USA 100:2969–2974
25. Kusano K, Fueshko S, Gainer H, Wray S 1995 Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. Proc Natl Acad Sci USA 92:3918–3922
26. Spergel DJ, Kruth U, Hanley DF, Sprengel R, Seeburg PH 1999 GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. J Neurosci 19:2037–2050
27. Bosma MM 1993 Ion channel properties and episodic activity in isolated immortalized gonadotropin-releasing hormone (GnRH) neurons. J Membr Biol 136:85–96
28. Costantin JL, Charles AC 1999 Spontaneous action potentials initiate rhythmic intercellular calcium waves in immortalized hypothalamic (GT1–1) neurons. J Neurophysiol 82:429–435
29. Van Goor F, Krsmanovic LZ, Catt KJ, Stojilkovic SS 2000 Autocrine regulation of calcium influx and gonadotropin-releasing hormone secretion in hypothalamic neurons. Biochem Cell Biol 78:359–370
30. Van Goor F, Krsmanovic LZ, Catt KJ, Stojilkovic SS 1999 Control of action potential-driven calcium influx in GT1 neurons by the activation status of sodium and calcium channels. Mol Endocrinol 13:587–603
31. Xu C, Xu XZ, Nunemaker CS, Moenter SM 2004 Dose-dependent switch in response of gonadotropin-releasing hormone (GnRH) neurons to GnRH mediated through the type I GnRH receptor. Endocrinology 145:728–735
32. Stojilkovic SS, Catt KJ 1995 Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 7:739–757
33. Merelli F, Stojilkovic SS, Iida T, Krsmanovic LZ, Zheng L, Mellon PL, Catt KJ 1992 Gonadotropin-releasing hormone-induced calcium signaling in clonal pituitary gonadotrophs. Endocrinology 131:925–932

34. Charles AC, Hales TG 1995 Mechanisms of spontaneous calcium oscillations and action potentials in immortalized hypothalamic (GT1–7) neurons. J Neurophysiol 73:56–64
35. Costantin JL, Charles AC 2001 Modulation of Ca(2+) signaling by K(+) channels in a hypothalamic neuronal cell line (GT1–1). J Neurophysiol 85:295–304
36. Van Goor F, Krsmanovic LZ, Catt KJ, Stojilkovic SS 1999 Coordinate regulation of gonadotropin-releasing hormone neuronal firing patterns by cytosolic calcium and store depletion. Proc Natl Acad Sci USA 96:4101–4106
37. Cesnjaj M, Krsmanovic LZ, Catt KJ, Stojilkovic SS 1993 Autocrine induction of c-fos expression in GT1 neuronal cells by gonadotropin-releasing hormone. Endocrinology 133:3042–3045
38. Yuen T, Wurmbach E, Ebersole BJ, Ruf F, Pfeffer RL, Sealfon SC 2002 Coupling of GnRH concentration and the GnRH receptor-activated gene program. Mol Endocrinol 16:1145–1153
39. Romanelli RG, Barni T, Maggi M, Luconi M, Failli P, Pezzatini A, Pelo E, Torricelli F, Crescioli C, Ferruzzi P, Salerno R, Marini M, Rotella CM, Vannelli GB 2004 Expression and function of gonadotropin-releasing hormone (GnRH) receptor in human olfactory GnRH-secreting neurons: an autocrine GnRH loop underlies neuronal migration. J Biol Chem 279:117–126
40. Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1–10
41. Bonner RF, Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA 1997 Laser capture microdissection: molecular analysis of tissue. Science 278:1481–1483
42. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA 1996 Laser capture microdissection. Science 274:998–1001
43. Grynkiewicz G, Poenie M, Tsien RY 1985 A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
44. DeVita RJ, Walsh TF, Young JR, Jiang J, Ujjainwalla F, Toupence RB, Parikh M, Huang SX, Fair JA, Goulet MT, Wyvratt MJ, Lo JL, Ren N, Yudkovitz JB, Yang YT, Cheng K, Cui J, Mount G, Rohrer SP, Schaeffer JM, Rhodes L, Drisko JE, McGowan E, MacIntyre DE, Vincent S, Carlin JR, Cameron J, Smith RG 2001 A potent, nonpeptidyl 1H-quinolone antagonist for the gonadotropin-releasing hormone receptor. J Med Chem 44:917–922
45. Urban RJ, Johnson ML, Veldhuis JD 1989 *In vivo* biological validation and biophysical modeling of the sensitivity and positive accuracy of endocrine peak detection. I. The LH pulse signal. Endocrinology 124:2541–2547

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